REMARKS

The parent application (U.S. Patent Appln. Ser. No. 09/314,847, filed May 19, 1999, now U.S. Patent No. 6,365,410) was originally filed with 48 Claims. Patent No. 6,365,410 contains Claims that correspond to Claims 1-3, 11-24, and 28-48 of the originally filed application. In the transmittal letter that was filed with the present Divisional application, Applicants requested that Claims 1-3, 11-24, and 28-48 be cancelled. As this request was not entered, Applicants submit that at the time of the present Restriction Requirement, all 48 Claims were pending.

In the present Restriction Requirement, the Examiner has restricted the Claims into five Groups as follows:

Group I:	Claims 1-39, drawn to methods for preparing an evolved microorganism;
Group II:	Claims 40-45, drawn to expression vectors and host cells comprising a mutator gene;
Group III:	Claim 46, drawn to a deposited microorganism;
Group IV:	Claim 47, drawn to a deposited microorganism; and
Group V:	Claim 48, drawn to a method for preparing an evolved microorganism.

As Applicants believe that Claims 1-3, 11-24 and 28-48 were addressed in the parent case, Applicants have cancelled Claims 1-3, 11-24 and 28-48. Applicants have also cancelled Claims 4-10, and 25-27, and added new Claims 49-58, which correspond to cancelled Claims 4-10 and 25-27. These new Claims find more than sufficient support in the Specification and Claims as filed and no new matter is added. As these Claims are within Group I, as designated by the Examiner, Applicants hereby elected the Claims in Group I, without traverse. Applicants reserve the right to pursue any or all of the cancelled Claims or similar Claims in one or more continuation application(s).

Applicants have also amended the Specification to provide the ATCC accession numbers for the deposited microorganism. Additionally, the SEQ ID NOs have been corrected. No new matter is introduced by these amendments.

Should the Examiner have any questions regarding this application, he is encouraged to call the undersigned.

Respectfully submitted,

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APPENDIX I

MARKED-UP VERSION OF SPECIFICATION'S REPLACEMENT PARAGRAPHS AND REWRITTEN, ADDED, AND/OR CANCELLED CLAIMS

The following is a marked-up version of the Specification's replacement paragraphs pursuant to 37 C.F.R. §1.121(b), as well as a marked-up version of the Claims pursuant to 37 C.F.R. §1.121 (c)(1)(ii) with instructions and markings showing changes made herein to the previous version of record of the Specification and Claims. Underlining denotes added text while bracketing denotes deleted text.

IN THE SPECIFICATION:

The paragraph on page 5, lines 10–17, has been amended as follows:

The present invention encompasses methods for evolving gram positive and gram negative microorganisms as well as yeast, fungus and eucaryotic cells including hybridomas. In one embodiment, the gram negative microorganism includes members of *Enterobacteriaceae* and in another embodiment comprises *Eschericia* and in another embodiment comprises *E.coli* and *E.blattae*. In further embodiments of the present invention, the evolved microorganism includes *E.coli* having ATCC accession number PTA-91 and *E.blattae* having ATCC accession number PTA-92.

The table on page 6, lines 10-13, has been amended as follows:

Depositor Identification Reference	International Depository Designation	Date of Deposit
Escherichia coli MM294 derivative	ATCC PTA-91	May 1[7]9, 1999
Escherichia blattae 33429 derivative	ATCC PTA-92	May 1[7]9, 1999

The use of a plasmid comprising a mutator gene, ie, a mutator plasmid, can be used to control the mutation rate of a microorganism. As described under Section II below, plasmid constructs can be designed which provide reduced levels of expression of a mutator gene thereby providing a means for altering the ratio of naturally occurring DNA repair genes vs mutator genes. This provides a means for combining the advantage of mutD mutations (which

results in random mutagenesis) with the advantages of the other known mutators (lower mutation frequency which leads to a lower burden on the cells). Additionally, plasmid constructs can be designed that allow for curing the evolved microorganism of the mutator gene, such as the use of a temperature sensitive origin, thereby allowing for a means for turning the mutation events off and on in the microorganism. For a gram positive microorganism, such as *B. subtilis* [B.subtilis] where the entire genome has been sequenced, the present invention could encompass the steps of deleting or mutating a DNA repair gene, evolving the *Bacillus* [Bacillus], and restoring the naturally occurring DNA repair system through recombination events. As disclosed herein, several members of [Escherichia] *Escherichia*, such as [E. coli and E. blatte] *E. coli* and *E. blattae* have been subjected to the directed evolution methods. Illustrative examples of evolved [E.coli and E.blattae] *E. coli* and *E. blattae* have been deposited with the ATCC and have accession numbers [_____ and _____] PTA-91 and PTA-92, respectively.

The paragraph beginning at page 26, lines 5-15, has been amended as follows:

The evolution of 1,3-propanediol resistance was faster in the presence of B12. After 2 months of evolution GEB025 (+B12) was able to grow with 95-100g/l 1,3-propanediol. After 3 months of anaerobic growth under selection in the presence of 1,3-propanediol, GEB028 (-B12) could grow in medium supplemented with 110g/l 1,3-propanediol. Analysis of aerobic growth of GEB031 on LB plates supplemented with 85, 95, 105 and 115g/l 1.3-propanediol showed that cells produce much bigger colonies in the presence of 85g/l in comparison with 105g/l. No growth was observed at 115g/l 1,3 propanediol. The results indicate that after 3 months of applying directed evolutions techniques described herein to *E.blattae*, the tolerance to 1,3 propanediol was increased from 75 g/l to at least 105 g/l under aerobic conditions. The plasmid was cured from the GEB031 strain by growing at 41.5 degrees. An illustrative clone, GEB031-4 was deposited with the ATCC and has accession number PTA-92.

The paragraph beginning at page 26, lines 25-26, has been amended as follows:
Strains - Wild type ATCC 33429, *E.blattae* [E.blattae] comprising the mutant PDD as described in Example 4 and having ATCC accession number PTA-92.

Examples 6 and 6, beginning on page 27, line 30, through page 28, line 35, have been amended as follows:

Example 6: Cloning and sequencing the 1,3-propanediol dehydrogenase genes (*dhaT)* from *E. blattae.*

The *dhaT* genes were amplified by PCR from genomic DNA from *E. blattae* as template DNA using synthetic primers (primer 1 and primer 2) based on the *K. pneumoniae dhaT* sequence and incorporating an XbaI site at the 5' end and a BamHI site at the 3' end. The product was subcloned into pCR-Blunt II-TOPO (Invitrogen). The cloning *dhaT* were then sequenced [was] with standard techniques.
The results of the DNA sequencing are given in SEQ ID NO:[1] 3 and SEQ ID NO:[2] 4.

Primer 1
5' TCTGATACGGGATCCTCAGAATGCCTGGCGGAAAAT3 ' SEQ ID NO:14

Primer 2
5' GCGCCGTCTAGAATTATGAGCTATCGTATGTTTGATTATCTG3' SEQ ID NO:15

As will be readily understood by the skilled artisan, nucleic acid sequence generated via PCR methods may comprise inadvertent errors. The present invention also encompasses nucleic acid encoding PDD obtainable from E.blattae having ATCC accession number [____.] PTA-92.

Example 7: Comparison of wild-type *E.blattae* (ATCC accession number 33429) and the evolved strain GEB031-4 (ATCC accession number [_____.)] PTA-92).

This example shows that *E.blattae* subjected to the methods of the present invention and having ATCC accession number [_____] PTA-92 can completely consume 800mM glycerol during anaerobic fermentation and does not accumulate 3-hydroxy-propionaldehyde (3HPA) and does not lose viability. In contrast, the wild-type *E.blattae* accumulates 50mM 3 HPA and becomes non viable after consuming only 350 mM glycerol.

The wild-type *E.blattae* and the evolved *E.blattae* were subjected to fermentation in the following medium: 75 g glycerol, 5 g $K_2HPO_4 \cdot 3H_2O$, 3 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 4 mg $CoCl_2 \cdot 2H_2O$, 2 g yeast extract, and 1 g peptone per liter water. The pH was maintained with 20% NaOH. Both fermentations were run at 30°C with a N_2 sparge and were inoculated with a stationary grown overnight preculture.

IN THE CLAIMS:

Please cancel Claims 1-48.

Please add the following new Claims:

49. A method for preparing an evolved microorganism comprising the steps of:

a) obtaining a microorganism comprising at least one heterologous mutator gene and at least one introduced nucleic acid encoding at least one heterologous protein, wherein said at least one heterologous protein is an enzyme;

b) culturing said microorganism for at least 20 doublings under conditions suitable for selection of an evolved microorganism, wherein said heterologous mutator gene generates a mutation rate of at least 5-100,000 fold relative to wild type; and

c) restoring said evolved microorganism to a wild type mutation rate.

50. The method of Claim 49, wherein said at least one heterologous protein is a hydrolase.

51. The method of Claim 50, wherein said hydrolase is selected from the group consisting of proteases, esterases, lipases, phenol oxidase, permeases, amylases, pullulananses, cellulases, glucose isomerase, laccases, and protein disulfide isomerases.

52. The method of Claim 49, wherein said microorganism comprises at least one copy of said mutator gene in its chromosome and said step of restoring said evolved microorganism to wild-type mutation rate comprises excision of said mutator gene.

53. The method of Claim 52, wherein said mutator gene comprises at least one gene selected from the group consisting of *mutD*, *mutT*, *mutY*, *mutM*, *mutH*, *mutL*, *mutS*, *mutU*, *mutD* mutations, *mutT* mutations, *mutY* mutations, *mutM* mutations, *mutH* mutations, *mutL* mutations, *mutS* mutations, *mutU* mutations, and homologues of *mutD*, *mutT*, *mutY*, *mutM*, *mutH*, *mutL*, *mutS*, and *mutU*.

54. The method of Claim 52, wherein said mutator gene comprises *mutD* mutations selected from the group of *mutD* mutations set forth in Table 1.

55. A method for preparing an evolved microorganism comprising the steps of:

a) obtaining a microorganism comprising at least one heterologous mutator gene and at least one introduced nucleic acid encoding at least one heterologous protein, wherein said at least one heterologous protein is an enzyme necessary for an enzymatic pathway;

b) culturing said microorganism for at least 20 doublings under conditions suitable for selection of an evolved microorganism, wherein said heterologous mutator gene generates a mutation rate of at least 5-100,000 fold relative to wild type; and

c) restoring said evolved microorganism to a wild type mutation rate.

56. The method of Claim 55, wherein said enzyme is selected from the group consisting of reductases and dehydrogenases, and further wherein said enzymatic pathway results in the production of at least one compound selected from the group consisting of ascorbic acid or ascorbic acid intermediates.

57. The method of Claim 55, wherein said enzyme is selected from the group consisting of glycerol dehydratase and 1,3-propanediol dehydrogenase, and further wherein said enzymatic pathway results in the production of at least one compound selected from the group consisting of 1,3-propanediol, 1,3-propanediol precursors, and 1,3-propanediol derivatives.

58. The method of Claim 55, wherein said enzyme is selected from the group consisting of glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatases, and further wherein said enzymatic pathway results in the production of at least one compound selected from the group consisting of glycerol and glycerol derivatives.